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An Opiate Cocktail that Reduces Morphine Tolerance and Dependence

Li He[1] and Jennifer L. Whistler[1,2,*]
[1]Ernest Gallo Clinic and Research Center
[2]Department of Neurology
University of California, San Francisco
5858 Horton Street, Suite 200
Emeryville, California 94608

Summary

Morphine is an exceptionally effective analgesic whose utility is compromised by the development of tolerance and dependence to the drug. Morphine analgesia and dependence are mediated by its activity at the mu opioid peptide (MOP) receptor [1]. The MOP receptor is activated not only by morphine, but also by other opiate drugs such as methadone and endogenous opioids such as endorphins. Morphine, however, is a unique opioid agonist ligand because it fails to induce endocytic trafficking of the MOP receptor [2], whereas the endogenous ligands and methadone do facilitate endocytosis [3]. Using the unique pharmacology of the MOP receptor and its proposed existence as an oligomeric structure [4], we designed a pharmacological cocktail that facilitates endocytosis of the MOP receptor in response to morphine. This cocktail consists of morphine and a small dose of methadone. Importantly, this cocktail, while retaining full analgesic potency, does not promote morphine dependence. We further demonstrate that dependence is reduced, at least in part, because endocytosis of the MOP receptor in response to morphine prevents the upregulation of N-methyl-D-aspartate (NMDA) receptors.

Results and Discussion

We found that a low dose of methadone facilitated the ability of morphine to induce MOP-receptor endocytosis both in HEK293 cells stably expressing an epitope-tagged version of the MOP receptor (Figures 1A and 1B) and in rat brain (Figures 1D and 1E). We utilized methadone, in particular the mixed *dl*-enantiomer of this drug, for these studies because it is widely available and inexpensive to produce, and the mixed enantiomer is the drug principally used clinically [5]. As shown previously [6], saturating doses of methadone (1 μM) promote endocytosis of the MOP receptor, whereas saturating doses of morphine (1 μM) do not (Figure 1A). We propose, then, that methadone- and morphine-occupied receptors are in different activated conformations, whereby the methadone-activated conformation better engages the endocytic machinery. We attribute the ability of a small number of *dl*-methadone-activated receptors to facilitate endocytosis of the morphine-activated receptors to the oligomeric nature of the opioid receptors [7]. Specifically, we propose that the MOP receptors exist as dimers/oligomers and that a single methadone-occupied receptor is sufficient to promote endocytosis of the dimer/oligomer complex when the other protomer(s) are occupied by morphine. This effect was observed with a subendocytic dose of *dl*-methadone in both the periaqueductal gray (PAG) (Figure 1D), a region critical for the analgesic properties of morphine [8], and the ventral tegmental area (VTA) (Figure 1E), a region critical for the rewarding properties of morphine [9].

Chronic morphine treatment of animals, as well as cells in culture, produces a compensatory upregulation of the cAMP pathway [10–12], a phenomenon termed cAMP superactivation. These elevated cAMP levels reflect cellular adaptive changes, which include increased expression of certain types of adenylyl cyclase, protein kinase A (PKA), and cAMP response element binding protein (CREB) (reviewed in [13, 14]). This phenomenon has been observed both in vitro and in brain regions implicated in addiction; these regions include the VTA [15], the locus coeruleus [11] and the striatum [16]. Importantly, these elevated levels of cAMP are responsible for changes in gene expression as well as for alterations in neurotransmitter release [14, 15, 17–19], making cAMP superactivation a hallmark of opiate tolerance and dependence.

Previously, we have demonstrated that receptor mutations that promote endocytosis of the MOP receptor in response to morphine can reduce this compensatory cAMP upregulation [20]. We therefore examined whether small doses of *dl*-methadone, which facilitated morphine-induced endocytosis, would likewise alter cAMP superactivation. As expected, morphine (1 μM) induced substantial cAMP superactivation (Figure 1C, gray bar, $p < .001$ versus no treatment). *dl*-methadone (10 nM) did not induce either receptor endocytosis (Figure 1A) or significant cAMP superactivation at this low dose (Figure 1C, striped bar). However, this low dose of *dl*-methadone (10 nM) substantially reduced cAMP superactivation in response to morphine (1 μM) (Figure 1C, black bar, $p < .001$ versus morphine alone), consistent with its ability to promote MOP-receptor endocytosis (Figures 1A and 1B).

We next examined whether a subendocytic dose of *dl*-methadone (50 nmol), which facilitated morphine-induced endocytosis in vivo (Figures 1D and 1E), could affect the development of either morphine tolerance or morphine dependence. First, rats were implanted with a catheter intracerebroventricularly (i.c.v.) through which drug or drug combinations were administered twice daily. We chose a morphine dose (30 nmol) that gave about 80% maximal possible effect (MPE) in the tail-flick test to insure that both decreases and increases in analgesia could be detected.

Rats treated with morphine alone developed profound tolerance by day 5 (Figure 2A, red line, open circle, $p < .001$ day 1 versus day 5). *dl*-methadone alone (50 nmol, black line, closed circle) produced no analgesia at this dose, nor did *dl*-methadone enhance acute morphine analgesia on day 1 (Figure 2A, compare red

*Correspondence: shooz2@itsa.ucsf.edu

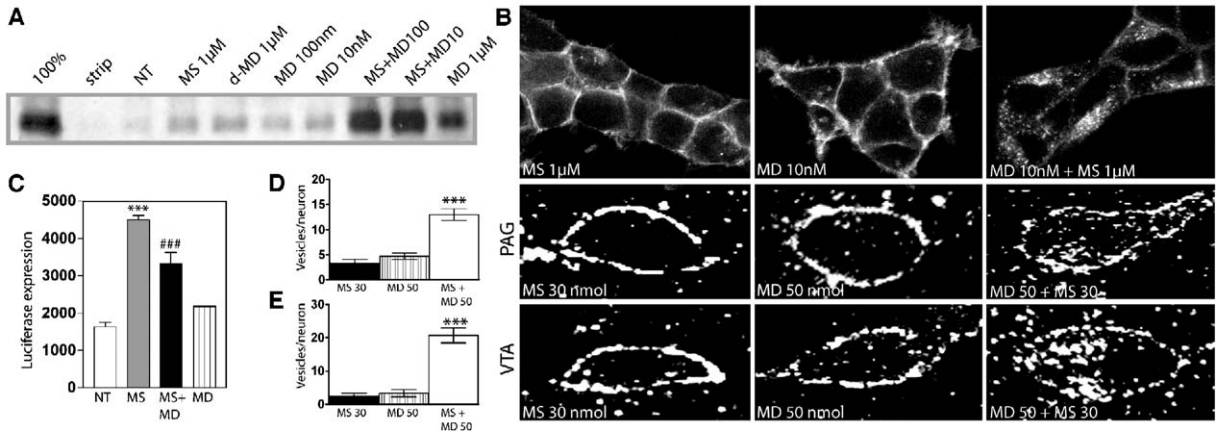

Figure 1. Trafficking and Superactivation of the MOP-R with Morphine and the Morphine-Methadone Cocktail

(A) Biotin protection assay to quantify endocytosis. HEK293 cells stably expressing FLAG-tagged MOP receptors were biotinylated with thioclavable biotin and treated with agonist/agonist combinations at stated concentrations or left untreated. Cells were stripped of remaining cell-surface biotin with membrane impermeant reducing agent, and "protected" receptors were immunoprecipitated and visualized (see the Supplemental Experimental Procedures in the Supplemental Data available with this article online). Methadone enhanced morphine-induced endocytosis. Saturating concentrations of morphine alone (1 μM) or *d*-methadone (1 μM) as well as low concentrations of *dl*-methadone (100 nM, 10 nM) failed to promote endocytosis. A saturating concentration (1 μM) of *dl*-methadone alone promotes endocytosis.

(B) Immunocytochemical staining. HEK293 cells stably expressing FLAG-tagged MOP receptors were fed antibody to the epitope tag and exposed to saturating concentrations of morphine (1 μM), 10 nM methadone, or both 1 μM morphine and 10 nM methadone, and then fixed and stained for receptor. Methadone enhanced morphine-induced MOP-receptor endocytosis.

(C) cAMP superactivation. CRE-luciferase expression was assessed in HEK293 cells stably expressing both the MOP receptor and a CRE-luciferase reporter gene [20]. Cells were treated with drug or drug combinations as listed for 14 hr and washed thoroughly, then treated with 2 μM forskolin for 4 hr. Morphine induced cAMP superactivation, whereas methadone decreased morphine-induced cAMP superactivation (*** $p < .001$ NT versus MS; ### $p < .001$ MS versus MS + MD, 1× ANOVA, Tukey post-test). The following abbreviations were used: MD, *dl*-methadone; MS, morphine sulfate; and NT, no treatment.

(D and E) Immunohistochemistry in rat brain. MOP-receptor distribution in PAG (D) and VTA (E) neurons of rats was assessed 30 min after acute i.c.v. administration of opiate drug or drug combinations. *dl*-methadone at 50 nmol or an analgesic dose of morphine (30 nmol) alone did not promote endocytosis of the MOP receptor, whereas coadministration of both drugs induced MOP-receptor endocytosis in neurons from both PAG and VTA. PAG denotes periaqueductal gray, and VTA denotes ventral tegmental area. For quantification, slides were encoded and vesicles counted by a second party from at least 10 cells from 2 rats per condition (*** $p < .001$: MS 30 nmol + MD 50 nmol versus MS 30 nmol or MD 50 nmol).

line, open circle and green line, open triangle). However, *dl*-methadone prevented the development of morphine tolerance at both the 50 nmol (Figure 2A, green line, open triangle) and also a lower 20 nmol dose (Figure 2A, blue line, closed square). A *dl*-methadone dose (5 nmol) that did not promote morphine-induced endocytosis of the MOP receptor in the rat brain (Figure 3) did not affect the development of morphine tolerance (Figure 2A, purple line, open square).

We have previously shown that enhanced endocytosis of the MOP receptor in response to morphine can reduce analgesic tolerance. However, the role of endocytosis in the development of morphine dependence has not been examined. Physical dependence on morphine is often associated with the development of tolerance, suggesting that these behavioral side effects of prolonged morphine use may be connected. However, these phenomena are not always observed together. For example, β-arrestin-2 knockout mice show reduced morphine tolerance but nevertheless develop morphine dependence [16]. We therefore assessed whether our opiate cocktail that reduced tolerance could also reduce morphine dependence. Indeed, *dl*-methadone, when coadministered with morphine, did reduce the development of morphine dependence, as measured by a significant decrease in the global score for naloxone-precipitated withdrawal signs (Figure 2B, $p < .01$ versus morphine alone). This effect was selective to methadone doses that facilitated morphine-induced endocytosis because very low doses of *dl*-methadone (5 nmol, Figure 2B, purple bar) had no effect on reducing the withdrawal signs.

To ensure that reduced tolerance and dependence were associated with enhanced endocytosis of the MOP receptor, we assessed the distribution of the MOP receptors in the brains of the rats that had been treated with drug or drug combinations for 5 days from experiment 2A. Morphine failed to induce MOP-receptor endocytosis even after 5 days of treatment (Figures 3A and 3B, left panels). Likewise, the 50 nmol dose of *dl*-methadone alone did not promote substantial endocytosis (Figures 3A and 3B). In contrast, MOP receptors in rats treated with the opiate cocktail of morphine 30 nmol + methadone 50 nmol were distributed not only on the plasma membrane, but also within intracellular compartments (Figures 3A and 3B, $p > .001$ versus morphine alone). The 5 nmol dose of methadone in combination with morphine did not promote endocytosis (Figures 3A and 3B, right panels), consistent with the inability of this dose to prevent tolerance and depen-

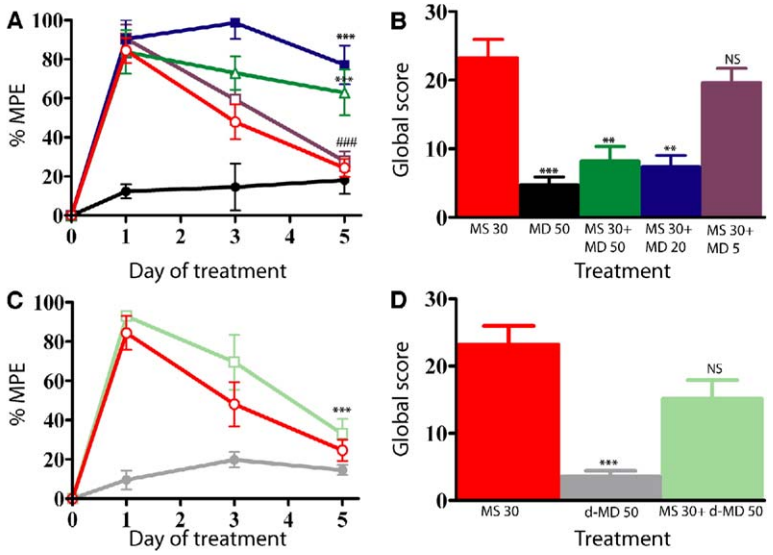

Figure 2. Tolerance to and Dependence on Morphine and the Morphine-Methadone Cocktail

(A and C) Analgesic tolerance. (A) Rats were implanted with an i.c.v. cannula, and morphine tolerance development was measured with a tail-flick assay over a 5 day period. Rats treated with an analgesic dose of morphine (30 nmol) developed substantial tolerance by day 5, whereas rats receiving the same dose of morphine plus varying doses of *dl*-methadone showed no tolerance at all but the lowest dose of methadone (*** $p < 0.001$: MS 30 nmol alone [red line, open circles] versus MS 30 nmol + MD 50 nmol [green line, open triangles] or MS 30 nmol + MD 20 nmol [blue line, closed squares]; ### $p < 0.001$: MS 30 nmol on day 5 versus day 1; MD 50 nmol alone [black line, closed circles]; MS 30 nmol + MD 5 nmol [purple line, open squares]). (C) Rats were implanted with an i.c.v. cannula, and morphine tolerance development was measured as above. Rats treated with morphine alone or morphine plus *d*-methadone (MS 30 nmol + *d*-MD 50 nmol [light-green line, open squares]) developed substantial tolerance (*** $p < 0.001$: day 5 versus day 1 MS 30 nmol + *d*-MD 50 nmol). *d*-methadone alone did not produce analgesia (gray line, closed circles). MPE denotes maximal possible effect (see Supplemental Experimental Procedures).

(B and D) Physical dependence. Rats from the tolerance experiments were injected with naloxone (3 mg/kg) subcutaneously (s.c.) after the final tail-flick assay on day 5, and withdrawal signs were monitored. (B) Withdrawal was significantly attenuated with several doses of *dl*-methadone compared with morphine alone (*** $p < 0.001$: MD 50 nmol versus MS 30 nmol; ** $p < 0.01$: MS 30 nmol + *dl*-MD 50 nmol versus MS 30 nmol; NS, no significance versus MS 30 nmol) (D) Withdrawal was not significantly attenuated by *d*-methadone (*** $p < 0.001$: *d*-MD 50 nmol versus MS 30 nmol; NS, no significance versus MS 30 nmol). For detailed statistics, please see Supplemental Experimental Procedures. The following abbreviations were used: MS, morphine sulfate; *dl*-MD, *dl*-methadone; and *d*-MD, *d*-methadone.

dence (Figures 2A and 2B). Together, these data strongly indicate a correlation between MOP-receptor trafficking and behavioral tolerance and dependence.

Methadone is a unique opioid drug in that it possesses not only MOP-receptor agonist activity but also antagonist activity at NMDA receptors [21]. This fact is highly relevant to this study because there is much evidence that the NMDA receptor system is involved in the manifestation of morphine tolerance and dependence (for review, see [22, 23]). Methadone is a mixed enantiomer of *d*- and *l*-methadone. *l*-methadone is the enantiomer primarily responsible for analgesia [24] and has a high affinity for the MOP receptor [25]. *d*-methadone has a low affinity for the MOP receptor [25], is a poor analgesic [26], and does not promote MOP-receptor endocytosis (Figure 1A). However, previously, *d*-methadone has been shown to prevent morphine tolerance [27], and this effect of *d*-methadone has been attributed specifically to the NMDA receptor antagonist properties of this enantiomer (k_d ~5–7 μM [28]). Although the methadone doses used in our studies were substantially lower than those reported to block tolerance, these observations raise the reasonable question of whether NMDA antagonism by *d*-methadone con-

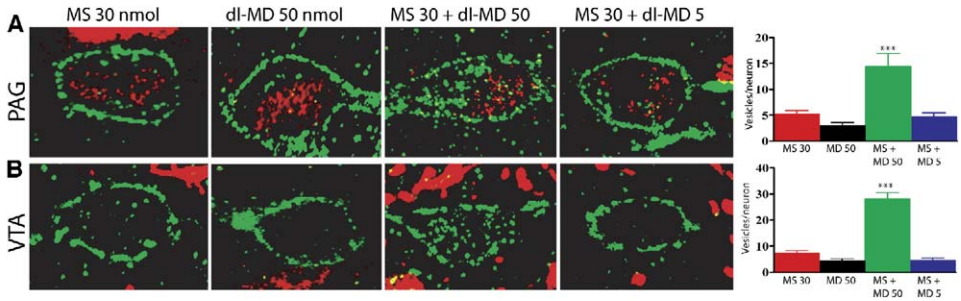

Figure 3. Immunohistochemical Staining of MOP Receptors in PAG and VTA Neurons of Rats from Figure 2A

MOP receptors (green) were primarily localized to the plasma membrane of neurons of rats treated with morphine at 30 nmol, *dl*-methadone at 50 nmol, and morphine 30 nmol + *dl*-methadone 5 nmol. Pronounced MOP-receptor endocytosis was found after coadministration of morphine 30 nmol + *dl*-methadone 50 nmol. For quantification, slides were encoded and vesicles counted by a second party from at least 10 cells from 2 rats per condition (*** $p < .001$ MS 30 nmol + MD 50 nmol versus MS 30 nmol, *dl*-methadone at 50 nmol, and morphine 30 nmol + *dl*-methadone 5 nmol). Red is NeuN in PAG and TH in VTA. Note that the MOP-receptor-positive cells are nondopaminergic neurons. The following abbreviations were used: PAG, periaqueductal gray; VTA, ventral tegmental area; TH, tyrosine hydroxylase; MS, morphine sulfate; *dl*-MD, *dl*-methadone; and *d*-MD, *d*-methadone.

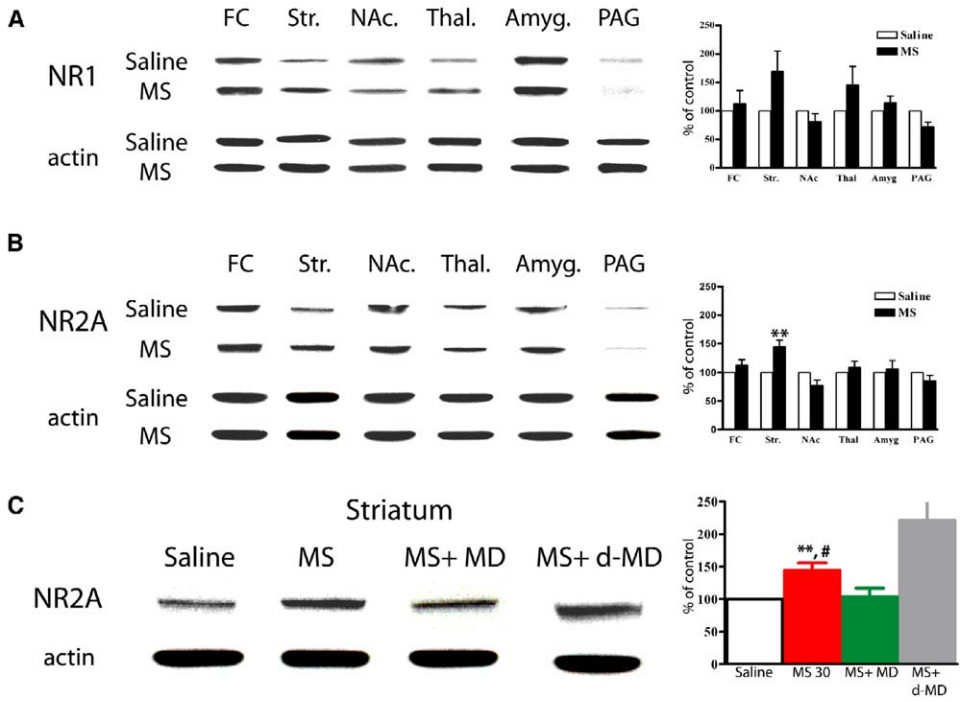

Figure 4. Effect of Drug Treatments on the Level of NMDA Receptor Subunits

(A–C) Protein levels were assessed in different rat brain regions by immunoblot. Data are expressed as mean ± SEM. Tissue samples were analyzed from two rats in each group, and at least two separate experiments were conducted for each rat. The following abbreviations were used: FC, frontal cortex; Str, striatum; NAc, nucleus accumbens; Thal, thalamus; Amyg, amygdala; and PAG, periaqueductal gray. ** $p < 0.01$, compared with saline group; # $p < 0.05$, compared with morphine plus *dl*-methadone group.

tributed to the anti-tolerance and/or -dependence effects of the morphine-methadone cocktail because the methadone utilized for our studies contains both the *d*- and *l*-enantiomers.

To examine this possibility, we utilized a morphine/*d*-methadone cocktail, which should retain any NMDA antagonism properties of our morphine/*dl*-methadone cocktail but not enhance MOP-receptor endocytosis. As expected, *d*-methadone (50 nmol) showed no analgesia (Figure 2C, gray line, closed circle), nor did it enhance acute morphine analgesia on day 1 (Figure 2C, green line, open square). Furthermore, at the dose administered in this study, *d*-methadone did not prevent morphine tolerance (Figure 2C, green line, open square) or dependence (Figure 2D). This strongly suggests that significant NMDA antagonism is not occurring at this methadone dose and that NMDA antagonism is not responsible for the ability of the mixed *dl*-methadone in our cocktail to prevent morphine tolerance and dependence. Although NMDA antagonists have been shown to block morphine tolerance and dependence (see below), taken together, our data suggest that it is the enhanced endocytosis of the MOP receptor, not NMDA antagonism, that is preventing morphine tolerance and dependence during administration of the cocktail.

We propose that endocytosis of the MOP receptor in response to morphine is reducing tolerance and dependence, at least in part, by reducing superactivation of the cAMP pathway (Figure 1; see [20]). However, there are several additional biochemical markers that have been found to be associated with morphine tolerance other than cAMP superactivation. These include redistribution of delta opioid receptors from intracellular compartments, where they normally reside, to the cell surface [29, 30] and upregulation of systems such as cholecystokinin and orphanin FQ/nociceptin (for review, see [31]).

One of the most intensely studied markers of morphine tolerance and dependence is the alteration of NMDA receptor number and function. Specifically, both the NR2A [32] and the NR1 [33] subunits of the NMDA receptor have been found to be upregulated after chronic morphine treatment. The fact that this NMDA receptor upregulation can be blocked by NMDA antagonists (for review, see [22]) could explain why NMDA antagonists, such as MK801 (k_d ~2 nM [21]) and *d*-methadone at high doses (k_d ~5–7 μM [28]), inhibit the development of morphine tolerance [33–35] and dependence [34]. Because activity at the MOP receptor has been shown to affect NMDA receptor function [36, 37], we investigated whether facilitating endocytosis of the MOP receptor influences morphine tolerance and dependence by altering the upregulation of the NMDA receptor.

First, we examined whether NR1 or NR2A were upregulated after the chronic morphine treatment utilized for our behavioral experiments. Expression of NR1 and NR2A (Figures 4A and 4B) was assessed in several brain regions after the behavioral experiments in Figure 2A. There was a trend toward upregulation of both NR1

and NR2A in several brain regions in response to morphine. However, the only statistically significant change was observed with the NR2A subunit in the striatum (Figure 4B), where expression was consistently increased by 50%. This observation is intriguing because the striatum is a brain region that shows significant cAMP superactivation in response to chronic morphine [16], suggesting these two phenomena may be related. In addition, previous studies have indicated that the striatum is more sensitive to changes in gene expression as a consequence of chronic morphine treatment than other anatomically closely related brain regions such as the nucleus accumbens [38], which might explain why we find the greatest changes in this region.

We next examined whether coadministration of *dl*-methadone (50 nmol), which facilitated endocytosis of the MOP receptor (Figures 1C and 1D) and prevented tolerance and dependence (Figures 2A and 2B), affected the upregulation of NR2A. Indeed, rats treated with morphine together with *dl*-methadone (50 nmol) did not show upregulation of NR2A (Figure 4C, compare lanes 1 and 3), suggesting *dl*-methadone prevented morphine-induced upregulation of NR2A. Importantly, coadministration of morphine together with *d*-methadone (50 nmol) did not prevent NR2A upregulation (Figure 4C, lane 4), consistent with the inability of *d*-methadone to prevent morphine tolerance and dependence (Figures 2C and 2D).

Together, these results suggest that small, subendocytic doses of methadone, when combined with morphine, facilitate endocytosis and thereby prevent cellular adaptive changes, including cAMP superactivation and NR2A upregulation, associated with chronic morphine use. These adaptive changes, in turn, likely combine to manifest in behavioral tolerance and dependence. Therefore, we propose that an opiate cocktail that combines morphine with a small dose of methadone would increase the effectiveness of morphine for the treatment of chronic pain. In addition, our results suggest that patients requiring prolonged opioid treatment would have a reduced liability for opioid dependence when treated with a cocktail of morphine and methadone.

Supplemental Data

Detailed Experimental Procedures and a supplemental figure are available at http://www.current-biology.com/cgi/content/full/15/11/1028/DC1/.

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